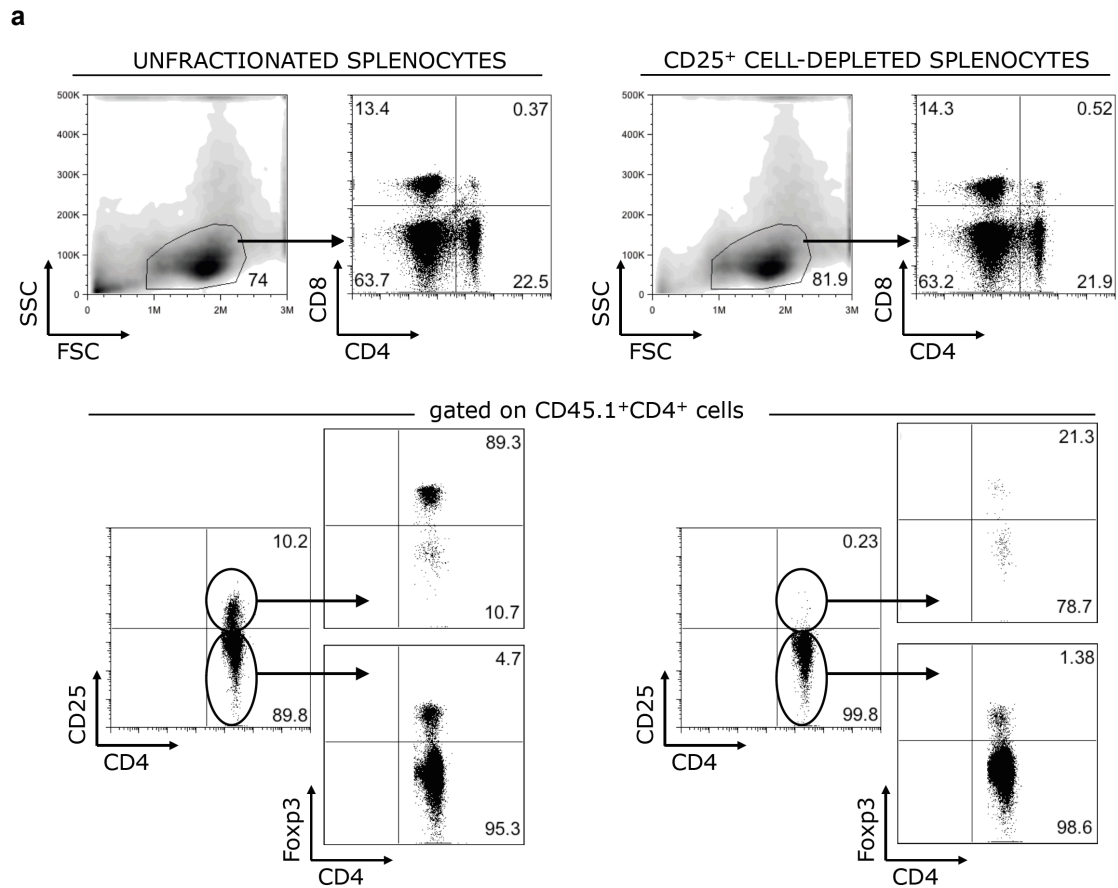


Supplemental Data from the original manuscript by Canto *et al.* entitled  
**Enlarged colitogenic T cell population paradoxically supports colitis  
prevention through the B-lymphocyte-dependent peripheral generation of  
CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells**

Fábio Barrozo do Canto, Sylvia M. N. Campos, Alessandra Granato, Rafael F. da  
Silva, Luciana Souza de Paiva, Alberto Nóbrega, Maria Bellio and Rita Fucs



**b**

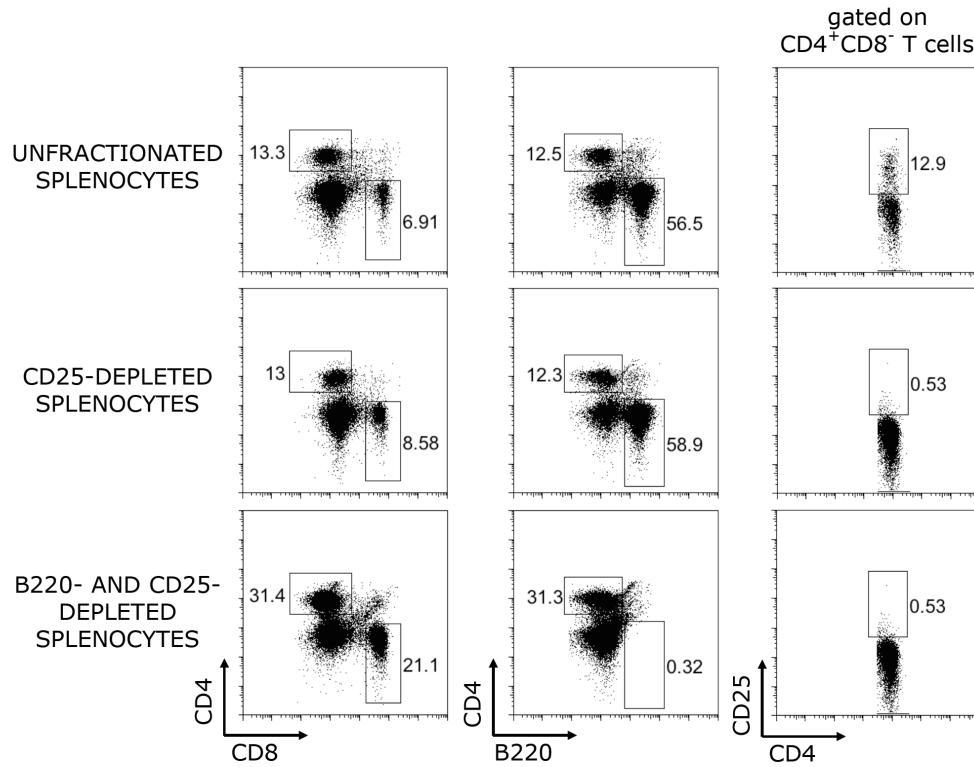
	Absolute numbers ( $\times 10^6$ ) of relevant T cell subsets present in the inocula				
	CD4 <sup>+</sup> CD8 <sup>-</sup>	CD4 <sup>-</sup> CD8 <sup>+</sup>	CD4 <sup>+</sup> CD25 <sup>-</sup> Foxp3 <sup>-</sup>	CD4 <sup>+</sup> CD25 <sup>-</sup> Foxp3 <sup>+</sup>	CD4 <sup>+</sup> CD25 <sup>+</sup> Foxp3 <sup>+</sup>
Unfractionated ( $20 \times 10^6$ )	$3.32 \pm 0.08$	$2.35 \pm 0.38$	$2.87 \pm 0.01$	$0.14 \pm 0.004$	$0.27 \pm 0.07$
CD25 <sup>-</sup> ( $20 \times 10^6$ )	$3.34 \pm 0.16$	$2.23 \pm 0.007$	$3.28 \pm 0.14$	$0.046 \pm 0.001$	$0.0025 \pm 0.001$
CD25 <sup>-</sup> ( $40 \times 10^6$ )	$6.7 \pm 0.32$	$4.6 \pm 0.02$	$6.56 \pm 0.31$	$0.092 \pm 0.003$	$0.005 \pm 0.003$

1

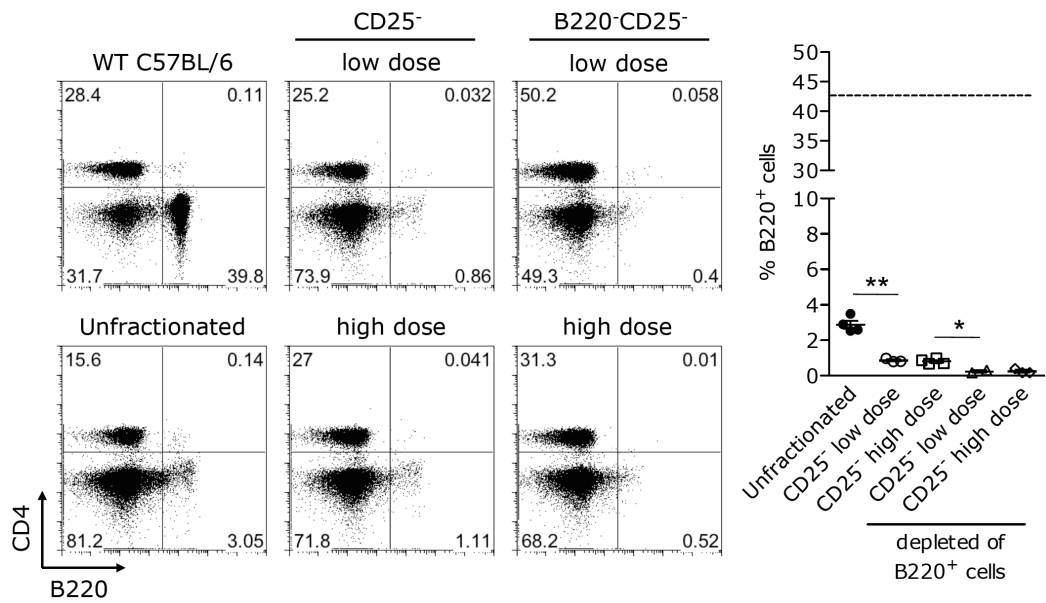
2 **Supplementary Figure 1: T cell frequencies are kept unaltered upon CD25<sup>+</sup>**  
3 **cell depletion and the absolute number of regulatory T cell contaminants**  
4 **in the inoculum is very low.** Flow cytometric characterization of the inocula of  
5 unfractionated and CD25-depleted splenocytes. (A) Representative dot-plots  
6 show the percentages of CD4, CD8 and CD25 T cells in adult B6.SJL  
7 splenocytes before (unfractionated) and after depletion of CD25<sup>+</sup> cells by  
8 magnetic-activated cell sorting. As depicted, CD25<sup>+</sup> cell depletion was very

1 efficient (> 99.0% pure) and did not affect single-positive CD4<sup>+</sup> or CD8<sup>+</sup> T cell  
2 proportions. Intracellular staining for Foxp3 shows the relative percentages of  
3 Foxp3-expressing cells within CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> subsets prior to *in*  
4 *vivo* inoculation of either unfractionated or CD25-depleted spleen cells. (B) Data  
5 from three independent CD25<sup>+</sup> cell depletions are pooled and the absolute  
6 numbers of relevant T cell subsets (mean ± SEM) present in each type of  
7 inoculum are shown.

a



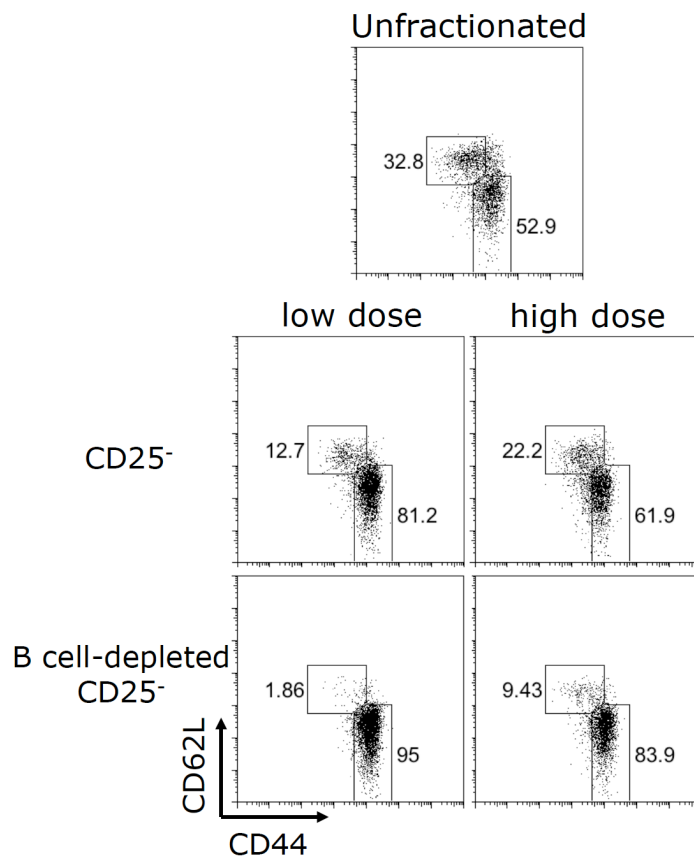
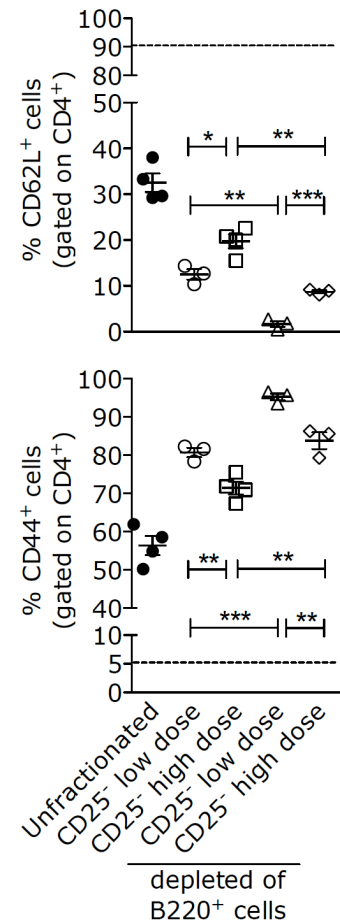
b



1

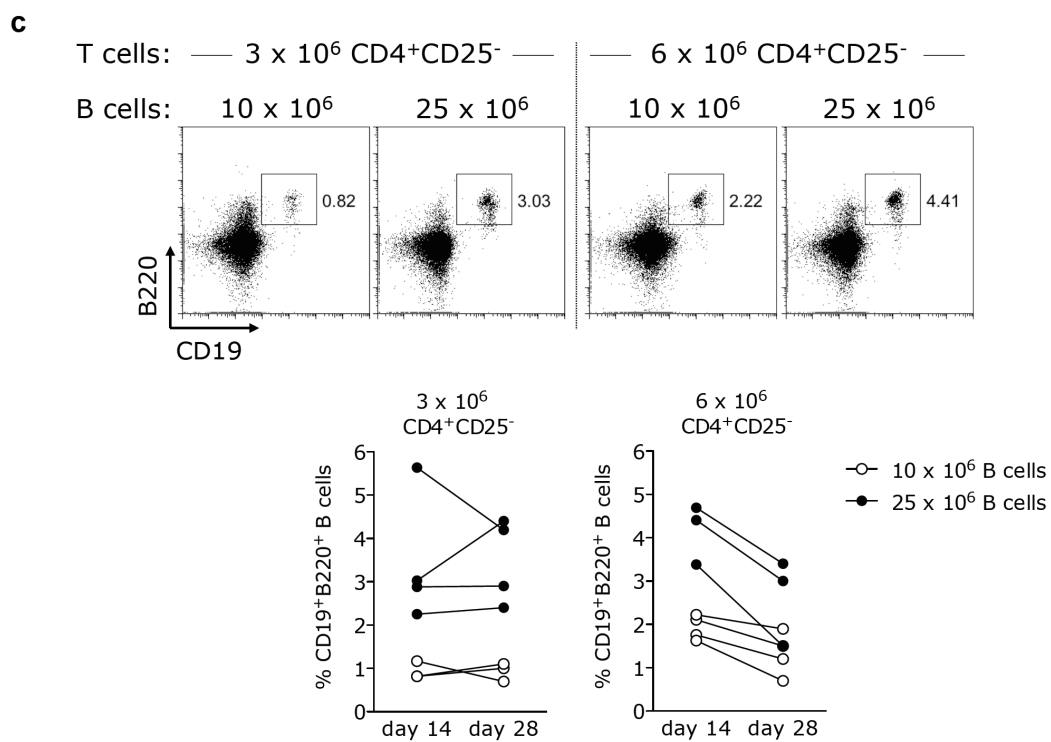
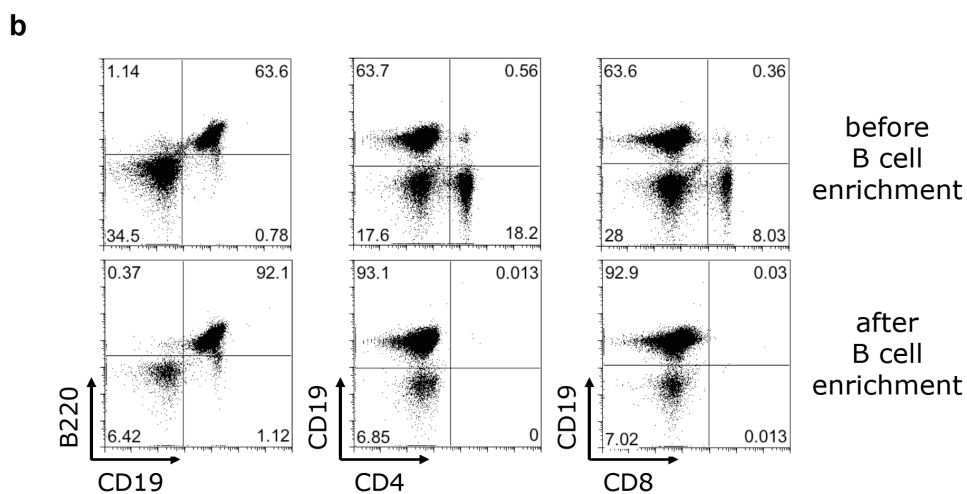
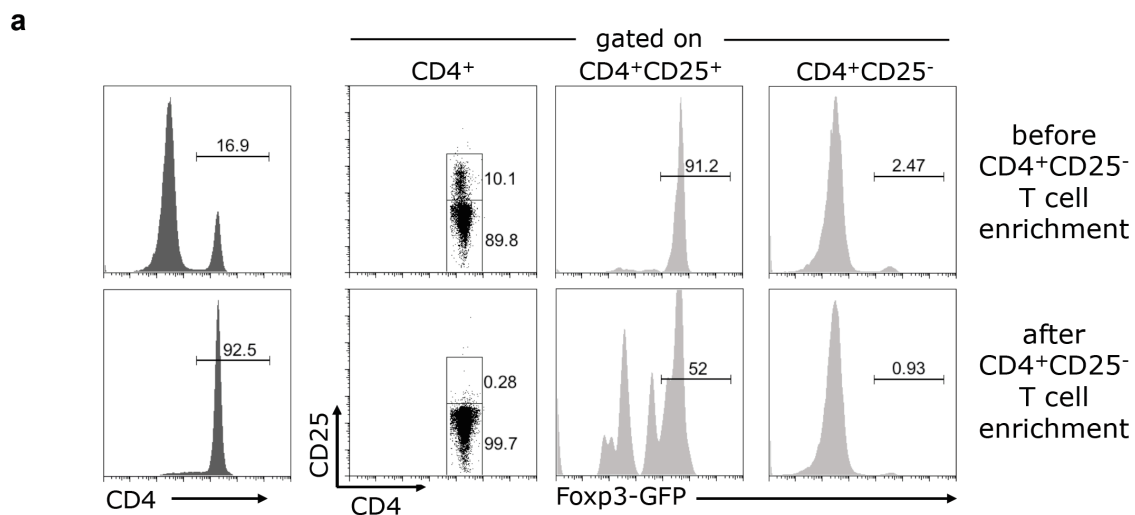
2 **Supplementary Figure 2: B lymphocytes are scarcely found in the**  
 3 **circulation of Rag<sup>-/-</sup> mice two weeks after reconstitution with B cell-**  
 4 **containing splenocytes. (A) Splenocytes obtained from adult C57BL/6 donors**  
 5 **were first depleted of CD25<sup>+</sup> cells and then incubated with biotin-conjugated**  
 6 **anti-B220 antibody for negative selection. B220<sup>-</sup>CD25<sup>-</sup> splenocytes were**

1 approximately 99.5% pure, showing virtually no B220<sup>+</sup> or CD25<sup>+</sup> contaminants.  
2 The selective elimination of B cells, and not of other minor B220-expressing cell  
3 populations, was confirmed by checking the purity of the fraction attached to the  
4 magnetic column, which was more than 99.0% positive for the exclusive B-cell  
5 lineage marker CD19 (not shown). MACS-based elimination of B220<sup>+</sup> cells  
6 resulted in two-fold increase on the representation of CD4<sup>+</sup> and CD8<sup>+</sup> T cell  
7 subsets in B220<sup>-</sup> splenocytes. Accordingly, 10 and 20 x 10<sup>6</sup> of B220<sup>-</sup>CD25<sup>-</sup>  
8 harbor about the same absolute numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes  
9 present in low (20 x 10<sup>6</sup>) and high (40 x 10<sup>6</sup>) dose of CD25<sup>-</sup> splenocytes and,  
10 therefore, were the doses chosen for injection. **(B)** B6.*Rag2*<sup>-/-</sup> mice were  
11 adoptively transferred with either unfractionated splenocytes, splenocytes  
12 depleted of CD25<sup>+</sup> cells (20 or 40 x 10<sup>6</sup> cells per mouse) or splenocytes  
13 depleted of both CD25<sup>+</sup> and B220<sup>+</sup> cells (10 or 20 x 10<sup>6</sup> cells per mouse)  
14 obtained from adult C57BL/6 donors and, two weeks after injection, the  
15 frequencies of B220<sup>+</sup> cells were determined in blood by flow cytometry. Dot-  
16 plots representative of one animal within each experimental group are shown.  
17 Numbers plotted in each quadrant refer to the percentage of events  
18 accumulated on lymphocyte gate established on the basis of FSC x SSC  
19 parameters. Data including all experimental animals in each condition (with at  
20 least three mice per group, mean ± SEM) are shown. Average B220<sup>+</sup> cell  
21 frequencies found in euthymic B6 mice are included for comparison (dashed  
22 horizontal lines). \*p < 0.05, \*\*p < 0.01.

**a****b**

**Supplementary Figure 3: B cells are required for the regulation of imbalanced CD62L/CD44 ratio within CD4<sup>+</sup> T compartment under lymphopenic settings.** Blood samples of mice described in *Figure 4* were collected 28 days after adoptive transfer and the frequencies of effector-like CD44<sup>high</sup>CD62L<sup>-</sup> and naïve CD44<sup>-</sup>CD62L<sup>high</sup> T cells were determined by flow cytometry. **(A)** Dot-plots representative of one animal within each experimental group are shown. Numbers plotted in each quadrant refer to the percentage of events accumulated on CD4<sup>+</sup>CD8<sup>-</sup> T lymphocyte gate. **(B)** Data including all experimental animals in each condition (with at least three mice per group, mean ± SEM) show blood frequencies of CD62L<sup>+</sup>CD44<sup>-</sup> (top) and CD44<sup>+</sup>CD62L<sup>-</sup> (bottom) relative to total CD4<sup>+</sup> T cells. Average frequencies of those CD4<sup>+</sup> T cell

- 1 subsets in naïve adult B6 mice are included for comparison (dashed lines). \*p <
- 2 0.05, \*\*p < 0.01, \*\*\*p < 0.001; not significant (ns): p > 0.05.
- 3



**Supplementary Figure 4: Enrichment of CD4<sup>+</sup> or B220<sup>+</sup> cells by MACS-based negative selection.** **(A)** Splenocytes isolated from adult C57BL/6.Foxp3-GFP donors were enriched for CD4<sup>+</sup> T cells accordingly to the protocol described in *Materials and Methods*. The flow-through was collected as an untouched CD4<sup>+</sup>CD25<sup>-</sup> T cell-enriched fraction (purity > 92%) and the percentages of CD25<sup>+</sup> and Foxp3<sup>+</sup> (determined on the basis of GFP fluorescence) cell contaminants are shown. **(B)** Splenocytes isolated from adult C57BL/6 donors were stained with biotin-conjugated antibodies for B220, CD8, CD11b, CD11c, CD49d and Tγδ and then placed on LD column for negative selection. The flow-through was collected as an untouched B cell-enriched fraction. Splenocytes enriched for B lymphocytes cells were > 92% pure and included no CD4- or CD8-expressing cells. **(C)** Blood samples of mice described in *Figure 4* were collected 14 and 28 days after adoptive transfer and the frequencies of donor-derived B220<sup>+</sup>CD19<sup>+</sup> B cells were determined by flow cytometry. Dot-plots representative of one animal within each experimental group show B cell frequencies 14 days after adoptive transfer. Numbers plotted in each quadrant refer to the percentage of events accumulated on lymphocyte gate established on the basis of FSC x SSC parameters. Data including all experimental animals in each condition (with at least three mice per group, mean ± SEM) show blood frequencies of B220<sup>+</sup>CD19<sup>+</sup> B cells determined 28 days after injection.